Mechanisms of Secondary Metabolite Production and Optimization of Antibiotic Synthesis by *Streptomyces griseus* Through the Use of Co-Cultures

An Honors Thesis (HONR 499)

By

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Abstract

Antibiotic synthesis by bacterial cultures is one of the most exploited biological pathways of the microbial world, and for good reason. All antibiotics that are currently used in modern medicine have their origins as by-products of bacterial secondary metabolism. Today, technology has allowed advances in areas of antibiotic research and development that have led to alterations and the production of semi-synthetic derivatives of antibiotics first isolated from bacterial cultures. The important of these antibiotic products cannot be understated in the treatment of infectious diseases, and as multidrug resistance rises in emerging pathogens, the continued discovery and isolation of new antibiotics from bacteria is ever important. This is why it is important to understand and analyze the mechanisms and optimal conditions that induce antibiotic production by various bacterial species. More specifically, the mechanism by which *Streptomyces griseus* is coaxed into producing the antibiotics streptomycin and cycloheximide, actual research methods aimed at optimizing antibiotic production by *S. griseus*, and future research induction of secondary metabolism are analyzed.

Acknowledgements

I would like to thank Dr. Mitchell for taking the time to advise me throughout this research project. His help talking through problems as well as procedural aspects during the laboratory research portion helped further my understanding of proper lab protocol.

I would like to thank Terry Bowser for my research project and this paper are extensions of his work here on his master's thesis. His research successfully defined the experimental and procedural protocols that were used throughout my research project.

Introduction

Microorganisms abundantly inhabit nearly every surface on the planet. In doing so, they inevitably face challenges ranging from harsh environments to invading microbes competing for the same nutrients and space. Bacteria and other microorganisms have colonized the Earth for billions of years, and as a result, have developed and inherited several adaptations to defend themselves against such stressors in their environment. As a domain, the genome of bacteria is incredibly malleable, and these microbes take advantage of this plasticity by readily acquiring DNA and adaptations from neighboring bacteria (Wilson et al. 2011). This allows entire bacterial populations to adapt to environmental shifts quickly. One beneficial adaption is their ability to turn to secondary metabolism in times of stress. Secondary metabolism is a state of growth induced by unfavorable environmental conditions in which the products of metabolism differ from ordinary growth in that they are meant to help the microbe outcompete and survive amongst other organisms or harsh conditions. One of the metabolic products that result from the induction of secondary metabolism is an antibiotic (Martin and Demain 1980). The production of an antibiotic in the presence of an invading neighbor microbe is done with the hope that the invading microbe is susceptible to the antibiotic and will therefore be eliminated by its production. There are many microbes that are capable of producing antibiotics as a secondary metabolite; one such microbe is *Streptomyces griseus*. In order to stimulate bacteria to produce an antibiotic secondary metabolite in a laboratory environment, another microbe, or challenge microbe, is placed in the same environment – this creates a co-culture. Using a co-culture with the original microbe plus a challenge microbe will increase the antibiotic or secondary metabolite synthesis (Pettit 2009). The optimization of this process is crucial because

nearly every antibiotic used in modern medicine originated or is a derivative of an antibiotic produced by a microbe. Therefore, the mechanisms that regulate secondary metabolism, the conditions that promote antibiotic production, laboratory techniques, and research methods involving co-cultures that promote and optimize the induction of secondary metabolism in *Streptomyces griseus* will be discussed in this paper.

Secondary Metabolism

Natural resources are at the forefront of competition between microbial species, and their availability can determine the dominating species in a community. There is a resource ration model of competition, which states that the dominate taxon of a particular environment depends on the availability of a limiting nutrient, the individual demand for that nutrient, and the rate of consumption of the nutrient (Hibbing et al. 2010). In other words, the species that predominates a particular niche will fluctuate with the availability of a particular nutrient. While specific nutrient availability may be a large factor in determining the microbial composition of a certain environment, the microbes themselves have the ability to tip the balance in their favor. Many species have developed mechanisms to enhance their nutrient acquisition in an effort to outcompete neighboring populations under stressful conditions (Hibbing et al. 2010). One mechanism employed by bacteria is going into a state of secondary metabolism induced by harsh conditions.

An important aspect to understand with regards to antibiotic production is secondary metabolism and its divergence from primary metabolism. A primary metabolite is a product of

primary metabolism, and is generally essential to life. These are produced under normal conditions, in an environment where the cell or organism is able to thrive. In contrast, a secondary metabolite is a product of metabolism that is not required by the organism for exponential growth. Secondary metabolites are produced in response to a stressful environment, and generally function to help the organism's survival (Martin and Demain 1980). It would seem that microbes are only programmed to produce secondary metabolites, such as antibiotics, under specific conditions such as when their growth rate drops below a particular level (Martin and Demain 1980). This mechanism was most likely produced to conservatively deal with competitive pressures seen in a natural environment where resources may be scarce, and the production of antibiotics can inhibit or kill any competing microorganisms.

In addition to the difference in function to the bacterium, primary and secondary metabolites are each produced at predictable stages in a bacterial population's growth, which follows a defined series of growth stages: the lag phase, exponential or log phase, stationary phase, and eventually death. The lag phase is the time period after initial inoculation of a culture in which there is no apparent growth or division. It is assumed that the bacterial cells are synthesizing and replicating the necessary proteins and enzymes in preparation for division during lag phase (Monod 1949). Exponential or log phase is the next period, and is marked by an exponential growth pattern as the cells divide via binary fission to double the colony size at a particular rate (Monod 1949). It is during this phase that a bacterial culture will produce its primary metabolites. Following the growth or exponential phase is the stationary phase in which division rate ceases. When microorganisms are placed in/on a particular medium, they reach a point at which they have exhausted available nutrients, experience an accumulation of

metabolic waste products, and/or run out of space for additional growth (Monod 1949). It is during the stationary phase in which microbes are challenged in their environment that they begin to produce secondary metabolites in an effort to combat the growth limiting factors they may experience. Antibiotic production occurs in stationary phase for reasons besides the developing stressful conditions of the phase – many of the species that produce antibiotics are actually susceptible to the antibiotic they produce with they are still growing (Martin and Demain 1980). Once the bacteria reach a certain point, they enter death phase. In this phase the number of viable cells drops as dramatically as they arose – at an almost exponential rate (Monod 1949). It is important to note that if growth of specified, antibiotic producing cultures is not stopped before this phase, the antibiotic will most likely be degraded. Microbial growth easily fluctuates depending on environmental conditions, and for clinical and research purposes, methods of promoting and extending stationary growth of a particular bacterium are essential to optimizing antibiotic yield.

Streptomyces griseus

A microorganism of specific interest for its antibiotic products is *Streptomyces griseus*. *S. griseus* typically inhabits soils as a saprophyte, degrading remains of other organisms (Challis 2003). It is the Gram positive soil bacterium that is responsible for the characteristic earthy smell of soil. As a bacterium that produces a mycelium, it more closely resembles the morphology seen by many filamentous fungi, and it is known for the production of aerial hyphae when grown on solid, or agar media (Bibb 2005). This bacterium is also non-motile,

which means that it had to evolve ways to combat nutrient limitations and competition rather than escape the stressors. As a result, *S. griseus* is largely responsible for many of the natural antibiotic products used today, even some anticancer metabolites (Wiley et al 2011). This particular characteristic makes it a popularly researched microorganism.

Streptomycin and its production by *S. griseus*

The actual initiation of secondary metabolism and production of the antibiotic is controlled genetically. The enzymes that induce antibiotic production are typically repressed during their microorganism's growth phase, and are only active when the growth rate drops below that predetermined level. In general, the enzymes that enable to the production of antibiotics are called antibiotic synthetases, and it is believed that the delayed production of antibiotics is due to the inhibition of these synthetases. It is believed that there are two enzymes important for the streptomycin biosynthesis pathway in *Streptomyces griseus*: amidinotransferase and streptidine kinase. Both of these were observed to be repressed during the organism's growth phase, but produced just prior to the production of streptomycin (Martin and Demain 1980). In addition to the streptomycin biosynthesis specific enzymes, there are a class of extracellular signals called γ-butyrolactones that are widely produced by *Streptomycetes* and are believed to play an important role in the onset of secondary metabolism; the γ-butyrolactone specific to *S. griseus* is known as A-factor (Bibb 2005). A-factor is an important inducer for stimulating the production of streptomycin by *Streptomyces griseus* (Martin and Demain 1980). Its role in the stimulation of the streptomycin biosynthesis pathway

is significant considering cultures only need to be exposed to A-factor for a few minutes, washed, and they are still able to produce streptomycin. Cultures that are not exposed to A-factor within forty-eight hours of inoculation are not stimulated to produce streptomycin (Martin and Demain 1980). It was also shown that exposure to this A-factor caused a significant number of mutated strains of *S. griseus* to regain the ability to produce streptomycin (Martin and Demain 1980). It was shown that this A-factor of *S. griseus* is actually required to bind to a cytoplasmic protein allowing for *adp*A transcription. The product, AdpA, is then required for the transcription of *str*R, which is the known regulatory gene of streptomycin production (Bibb 2005). Essentially, A-factor is required for the start of an activation pathway culminating in the production of streptomycin. In summary, it would seem that secondary metabolism and the production of antibiotics is the result of regulatory cascades. These cascades are initiated by a number of possible environmental signals, all leading to their own specific pathway and regulatory genes that control specific secondary metabolism products.

The antibiotic streptomycin first isolated from *Streptomyces griseus* is active against a variety of bacterial types including Gram negative and Gram positive bacteria as well as acid-fast bacteria. This antibiotic is ineffective in treating infections from anaerobic bacteria, fungi, or viruses (Waksman 1952). Additionally, streptomycin is actually most potent on young, actively dividing bacterial cultures rather than older cultures, but its activity is not completely diminished with older cultures as it is with some other antibiotics (Waksman 1952). Streptomycin originally received the most praise in its success in treating tuberculosis. The search for a treatment for tuberculosis is actually what sparked the investigation into the antibiotic secondary metabolites of *S. griseus* by Waksman.

The actual mechanism of streptomycin involves its inhibition of protein synthesis by inactivating the 30S ribosome thereby blocking the ribosome cycle (Luzzatto et al. 1968). Both the 30S and 50S ribosomal subunits are required for the production of proteins, but streptomycin is capable of diminishing the movement of ribosomes, meaning the two subunits would not be able to bind to an mRNA sequence and produce protein (Luzzatto et al. 1968). Streptomycin as a by-product of secondary metabolism in *S. griseus* was an important discovery as it has several medicinal uses today including its use as a viable alternative to pathogens that have become resistant to penicillin.

Cycloheximide and its production by *S. griseus*

Cycloheximide is a type of glutarimide antibiotic known for its antifungal properties. Essentially, the mode of action of cycloheximide involves the inhibition of protein synthesis via 80S ribosomes (Obrig et al. 1971). In utilizing the 80S ribosome, cycloheximide exerts its effects on protein synthesis by inhibiting peptide elongation and, more commonly, initiation via the donor ribosomal site on the 80S ribosome.

When *S. griseus* was tested to determine the conditions and mechanisms that control cycloheximide production, it was noted that utilization of glucose corresponded with start of cycloheximide production. In addition the exhaustion of glucose supplies also corresponded with the termination of cycloheximide production as well as the degradation of any cycloheximide that had accumulated up until that point (Kominek 1975). These observations seemingly conclude that glucose plays an apparent role in the production of cycloheximide by

Streptomyces griseus. In addition, cycloheximide itself plays a role in its biosynthesis pathway – the accumulation of cycloheximide most likely serves as a factor in the repression or feedback inhibition of the mechanism that controls cycloheximide production (Kominek 1975). This study also sought to show that the production of cycloheximide by *S. griseus* is a highly regulated mechanism in that it does not waste energy in the overproduction of the antibiotic. Increasing concentrations of cycloheximide were added to the flasks containing the cycloheximide producing *S. griseus*, and that rate of cycloheximide production was recorded. It was observed that cycloheximide production dropped dramatically with the increase of cycloheximide concentration added to the flask supporting the idea that *S. griseus* will not indulge in the overproduction of the antibiotic (Kominek 1975). Overall, it can be concluded that glucose concentrations directly correspond to the rate of synthesis of cycloheximide by *S. griseus* – maximum cycloheximide production corresponds to the greatest glucose concentration. The decrease in production also corresponds to the depletion of available glucose, and the degradation of the antibiotic occurs after complete exhaustion of glucose (Kominek 1975).

With the observation that the accumulation of cycloheximide has a negative correlation with its production, product (cycloheximide) removal while the microbe is still in culture was proposed to improve the yield of the secondary metabolite. This is done the hope to reduce the feedback regulation that leads to decreased cycloheximide production, removal of products to decrease toxicity rates, and to prevent the degradation of the antibiotic, which may occur of the bacteria is allowed grow in a stressed environment for too long (Payne and Wang 1989). Implications of evidence that the removal of the cycloheximide by-product will increase the efficacy of the rate at which *S. griseus* is able to convert glucose to cycloheximide include

reduced cost to profit margin when seeking the production of this antibiotic (Payne and Wang 1989). It is therefore proposed that in order to achieve the maximum cyclohexamide production, experimental protocols must be in place to either remove the antibiotic by-product or to stop its production at its peak.

The importance of cycloheximide cannot be understated for antibiotics with antifungal activity are not overly common. Since cycloheximide exhibits this activity, its use is not as common or as well researched as one of the other antibiotic products of *S. griseus*, streptomycin.

Experimental Techniques Using Co-Cultures

It was seen that *S. griseus* may be stimulated to produce both streptomycin and cycloheximide, which are both important antibiotics, so it is important to understand and discover methods to maximize the efficacy at which *Streptomyces griseus* is able to produce these two antibiotics. The use of specific challenge microbes in a co-culture with *Streptomyces griseus* can force it to produce certain antibiotics. Co-cultures are used to mimic a natural environment in which a microbe's environment may be invaded by a neighboring pathogen. Therefore, the use of co-cultures stimulates enhances the production of antibiotics by promoting secondary metabolism. In regards to co-cultures that enhance the production of secondary metabolites by *S. griseus*, a number have been identified, which include *Penicillium chrysogenum, Fusarium oxysporum, Rhizobium leguminosarum,* and the *Streptomycetes* isolates *Strepto*8 and *Strepto*Blue2 (Bowser 2013). In addition to co-cultures, defined media can be used to promote slower growth and production of antibiotics as a secondary metabolite

directly following inoculation. Defined medium is a growth media that is constructed for a particular microbe, and may contain only small amounts of a limiting nutrient to stress the microbe (Martin and Demain 1980).

While defined medium or stressed nutrient conditions may help in the coercion of the microbe to switch to secondary metabolism, the use of co-cultures in a production medium ensure that switch as well as direct the products of the microbe's secondary metabolism. Previous research has determined that the challenge microbe *Strepto*8 is capable of inducing the biosynthesis pathways of both streptomycin and cycloheximide, which made it a target for further study (Bowser 2013).

Materials and Methods

The method by which each of these microbes are grown, stimulated to produce antibiotics, and quantification of their antibiotic production is a lengthy and meticulous process as the microorganisms themselves as well as several of the tests employed are sensitive to even the slightest variation. Each of the microorganisms used in these experiments were first cultured on solid agar media, and an isolated colony from that culture is placed in liquid seed media flasks where they are allowed to grow for varying lengths. The see medium (ATCC medium #172) was (g/L): 10.0 glucose, 20.0 soluble starch, 5.0 yeast extract, 5.0 NZamine A, 1.0 $CaCO_3$. Flasks containing 50mL of this seed media were prepared, autoclaved, inoculated with the challenge microbe, and placed on a shaker at 210rpm for one week prior to the start of the

production media. Forty-eight hours before the start of the production media, *S. griseus* was started in a seed culture prepared the same way as before.

Following these required time periods, production media was prepared for inoculation. The production media, which was a modified Kominek, was comprised of (g/L): 60.0 glucose, 15.0 white bean flour, 2.5 yeast extract, 5.0 $(NH_4)_2SO_4$, 8.0 $CaCO_3$, 4.0 NaCl, 0.2 KH_2PO_4. This too was prepared, autoclaved, inoculated, and placed on a shaker at 210rpm for a week. *S. griseus* was first placed in the production media flasks, where secondary metabolism was induced, and allowed to grow for twenty-four hours before the introduction of a challenge microbe. The production flasks then went for a week from the initial inoculation with *S. griseus* before growth was stopped. This time frame was determined to be the ideal time to promote the greatest antibiotic production as well as ending the experiment before nutrients were exhausted and the antibiotics were degraded (Bowser 2013).

Antibiotic production is then assessed using bioassays and the Kirby-Bauer protocol for each antibiotic. The bioassays are conducted by first creating seeded agar plates with microorganism that are susceptible to cycloheximide and streptomycin – *Saccharomyces cerevisiae* and *Bacillus subtilis*, respectively. *Saccharomyces cerevisiae* was grown on PDA plates before being suspended in a 0.85% NaCl, or physiological saline solution. *B. subtilis*, was grown in TSB for 16 hours prior to pouring plates – tubes were vortexed often to prevent pellicle formation. The seeded agar plates were TGY agar: (g/L): 3.0 glucose, 3.0 tryptone, 3.0 yeast extract, 1.0 K_2HPO_4, and 15.0 agar to which either a $0.5\%_{(v/v)}$ yeast suspension or 2mL to 200mL of *B. subtilis* were added.

The Kirby-Bauer technique was then utilized to determine the diameter of inhibition zones of the treatment flasks, which were compared to a standard curve that was done at the same time (Bowser 2013). For each repetition for each treatment, 20µL were placed onto a 6mm sterile disc that was placed on the seeded agar plate, and allowed to incubate at their proper temperatures for twenty-four hours. For the cycloheximide assay, samples first had to be diluted to a 1:20 dilution before plating. This meant 30µL of the treatment sample was placed in 570µL of distilled water and 20µL of that solution was then placed on the 6mm sterile disc.

In order to determine the actual antibiotic concentrations that were produced from the antibiotics, a standard curve was run at the same time of the bioassay. A standard curve was run for both cycloheximide and streptomycin from known, purified stock solutions. A known concentration of 2000µg/mL was used to begin a 1:2 serial dilution until the concentration reached a satisfactory range ending with a 2^{-11} dilution. Following the dilution 20µL of each dilution was placed, in duplicate, on 6mm sterile paper discs and on their respective plates. It is important to run the standard curves on the same day as the treatments were plated to accurately determine the antibiotic concentrations because they will be adjusted to the same conditions.

The measured inhibition zone diameters were placed in the equation created by this graph to determine the cycloheximide concentrations of all the treatments. These concentrations were then used for further statistical analysis to determine whether co-cultures produced a significant increase in the production of cycloheximide. In order to measure any significant differences and qualify those differences into groups, the analysis of variance, or

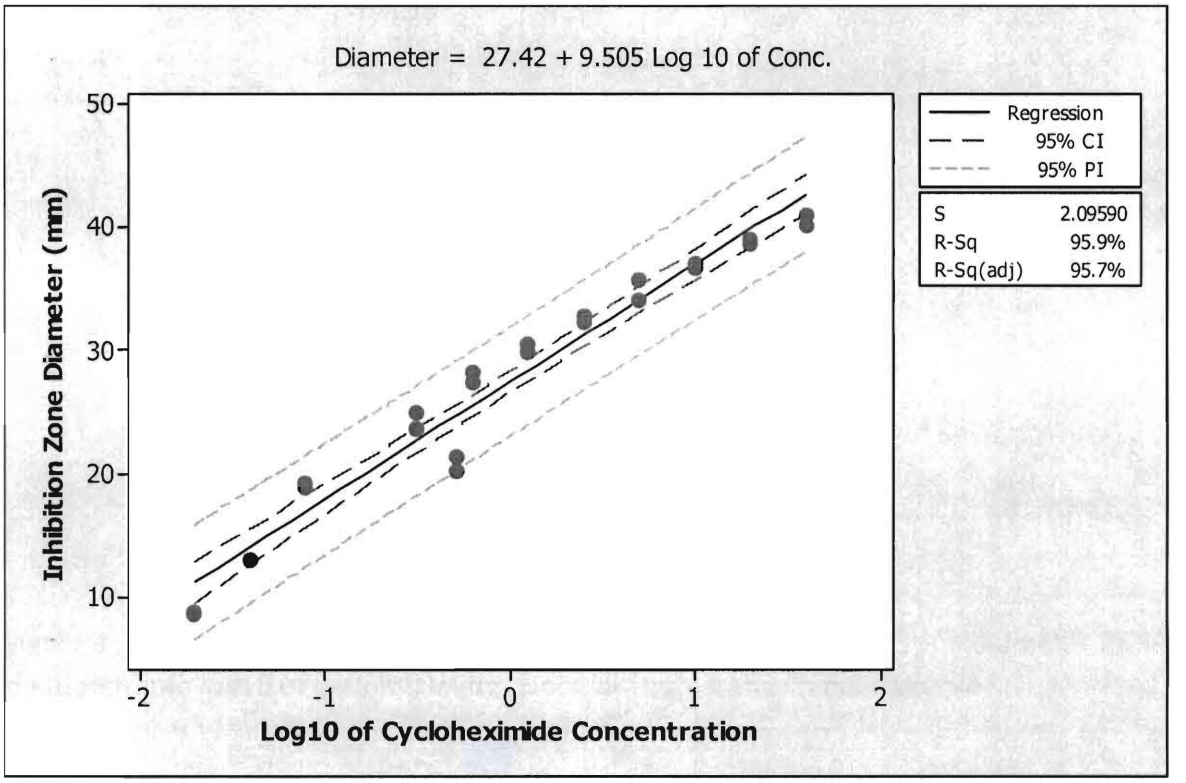
ANOVA, test was used. More specifically, the One-Way ANOVA in combination with the Tukey and Fisher/LSD tests to separate any significantly different treatments into groups. In order to perform these tests, the data collected must pass at least two other tests to determine whether the data itself meets the required assumptions to even perform an ANOVA test – normal distribution and equal variances.

The significance of this procedure is that it is a controlled experiment in which *Streptomyces griseus* is stimulated to produce antibiotics for a set time period. The bioassay protocol following that growth period allows for the quantification and direct comparison of the efficiency of antibiotic production under different treatment conditions because it is compared with a standard curve for each antibiotic. The effects of various co-cultures as wells as other possible treatments on the stimulation of antibiotic production can be assessed using this method, which will hold implications for optimized antibiotic production both experimentally and commercially.

Results

The data collected from the two standard curves was used to conduct their own separate linear regression analyses. From this linear regression analysis, a best fit line was created for cycloheximide, and the equation of that line was then used to relate inhibition zone diameter and antibiotic concentration allowing for the determination of the antibiotic concentrations created by each treatment group (Figure 1).

Figure 1. Fitted line plot from linear regression analysis of inhibition zone diameter (mm) in relation to the log10 of cycloheximide concentration in the standard curve



Once tests of normal distribution for each treatment group in cycloheximide production (Figures 2 and 3) and tests for equal variances (Figure 4) were passed, the One-Way ANOVA was conducted to analyze data from cycloheximide production for significant differences.

Figure 2. Probability plots of cycloheximide production by *S. griseus*, *Strepto8*, *StreptoBlue2*

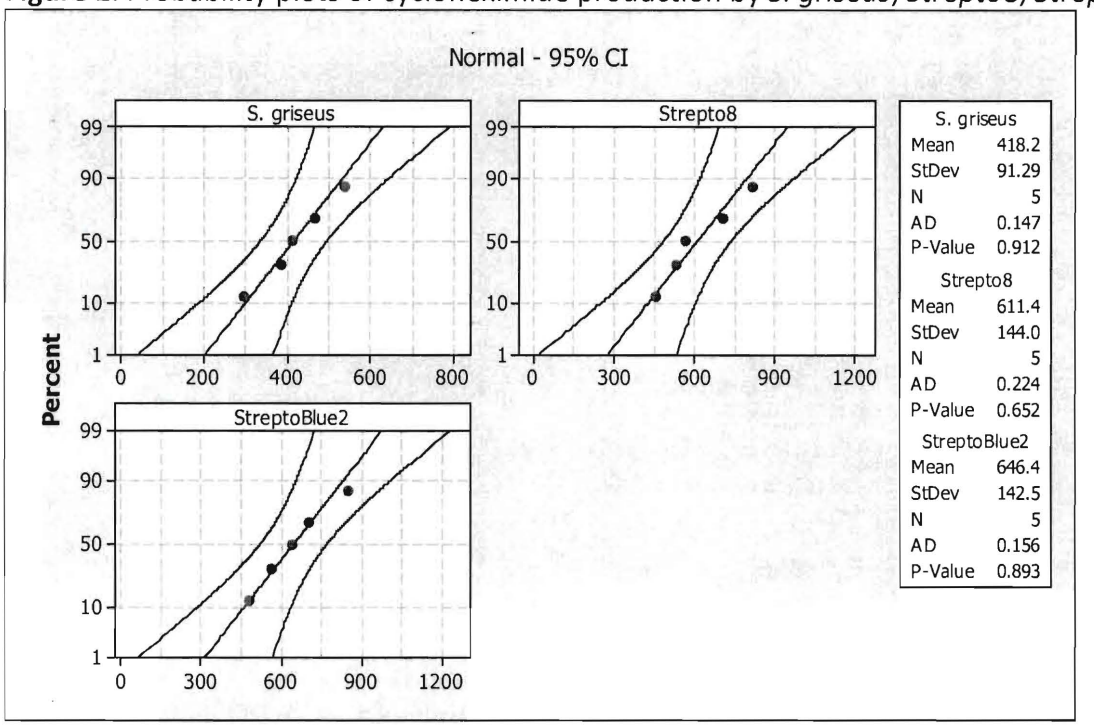
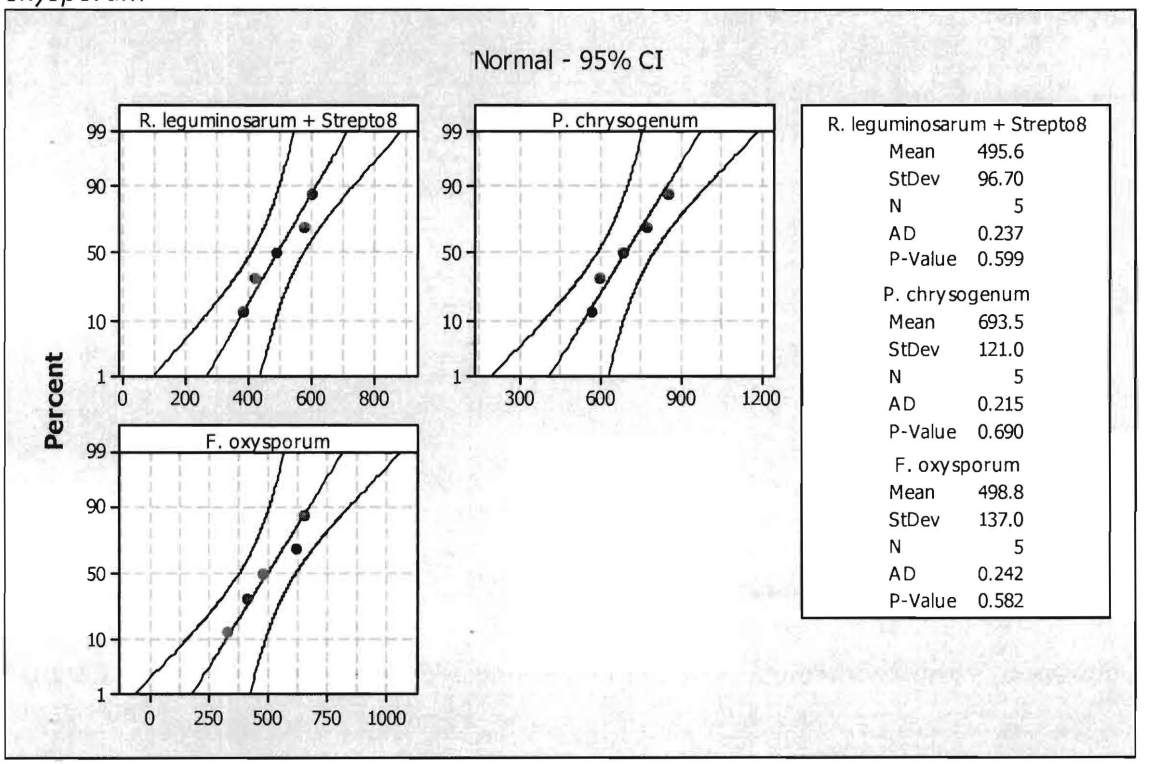
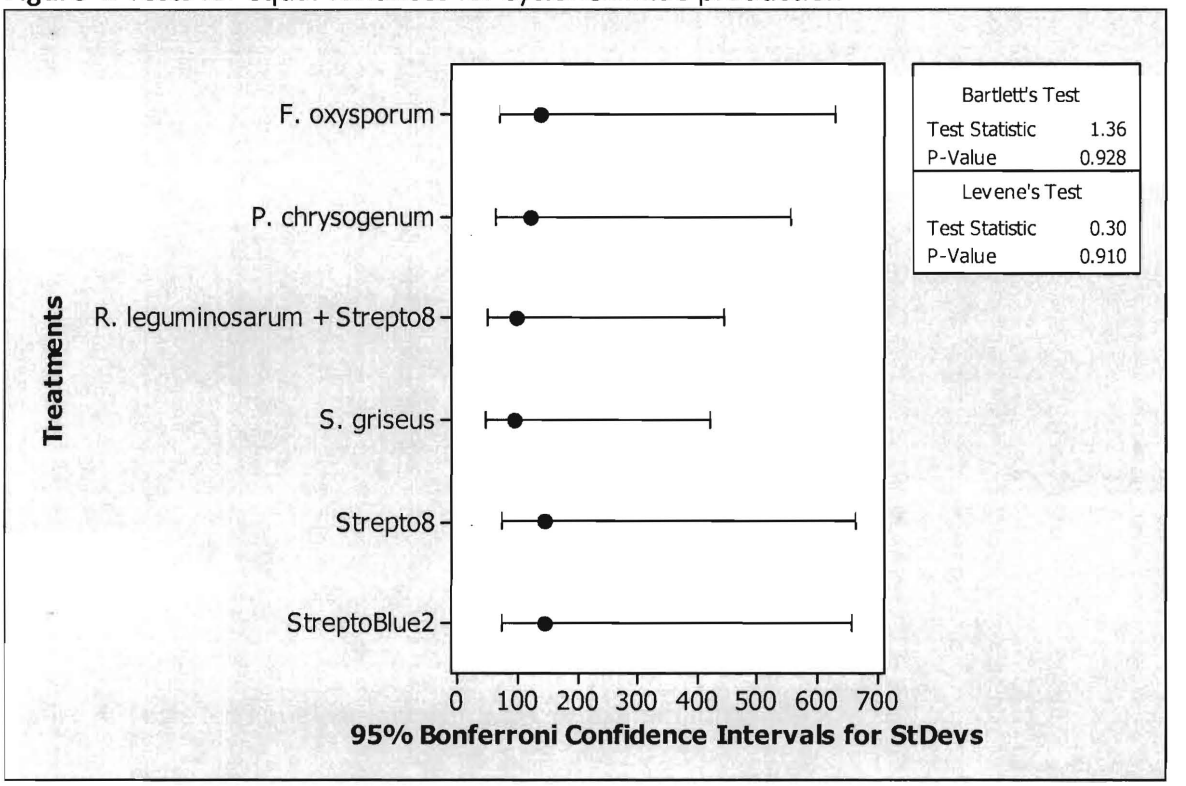


Figure 3. Probability plot of cycloheximide production *R. leguminosarum, P. chrysogenum, F. oxysporum*



*For P-values that are above 0.10 the data is considered to be normally distributed

Figure 4. Tests for equal variances for cycloheximide production



*The Bartlett's and Levene's tests both test for the Null hypothesis that all group variances are equal by providing a P-value that reflects the Null hypothesis.

The P-values provided in figure 4 are greater than 0.10, indicating equal variances, and that the assumptions for ANOVA have been met. The One-Way ANOVA test was now conducted using the data concerning cycloheximide production. The overall P-value for the One-Way ANOVA test conducted on cycloheximide production by *S. griseus* and various challenge microbes is 0.014, which indicated a strong likelihood that there will be at least two significantly different groups.

Table 1. Results of cycloheximide production by *S. griseus* + challenge microorganisms from One-Way ANOVA.

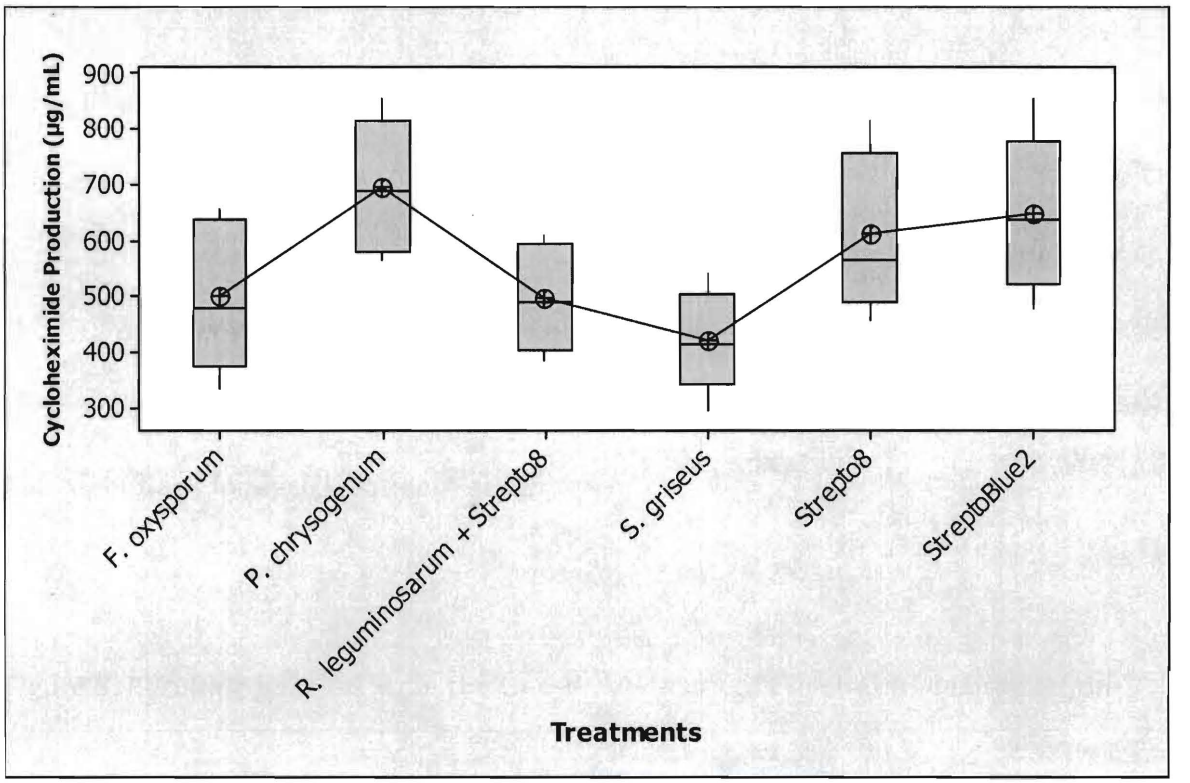
Treatment	N	Mean (µg/mL)	Tukey	Fisher/LSD
S. griseus + P. chrysogenum	5	693.5	A	A
S. griseus + StreptoBlue2	5	646.4	A B	A B
S. griseus + Strepto8	5	611.4	A B	A B
S. griseus + F. oxysporum	5	498.8	A B	B C
S. griseus + R. leguminosarum + Strepto8	5	495.6	A B	B C
S. griseus	5	418.2	B	C

*For Tukey and Fisher/LSD tests, each group with a different letter is significantly different ($P \leq 0.05$) from another

Results from One-Way ANOVA of cycloheximide production are shown in Table 1. When looking at any significant increases in cycloheximide production from the co-cultures compared to just *S. griseus*, the Fisher/LSD portion of the One-Way ANOVA show that *S. griseus + P. chrysogenum*, *S. griseus + StreptoBlue2*, and *S. griseus + Strepto8* all show a significant increase. While the *S. griseus + F. oxysporum* and *S. griseus + R. leguminosarum + Strepto8* co-cultures may show an increase in cycloheximide production, it is not large enough to be a significant increase from that of *S. griseus* alone (Table 1). Unlike the Fisher/LSD test, the Tukey test only shows that one co-culture, *S. griseus + P. chrysogenum*, showed significant cycloheximide increase from *S. griseus* (Table 1).

To better visualize cycloheximide production by the co-cultures used in this experiment, the boxplot shown in Figure 5 displays the average cycloheximide production from each of the five repetitions for each treatment group.

Figure 5. Resulting boxplots from the One-Way ANOVA of cycloheximide production

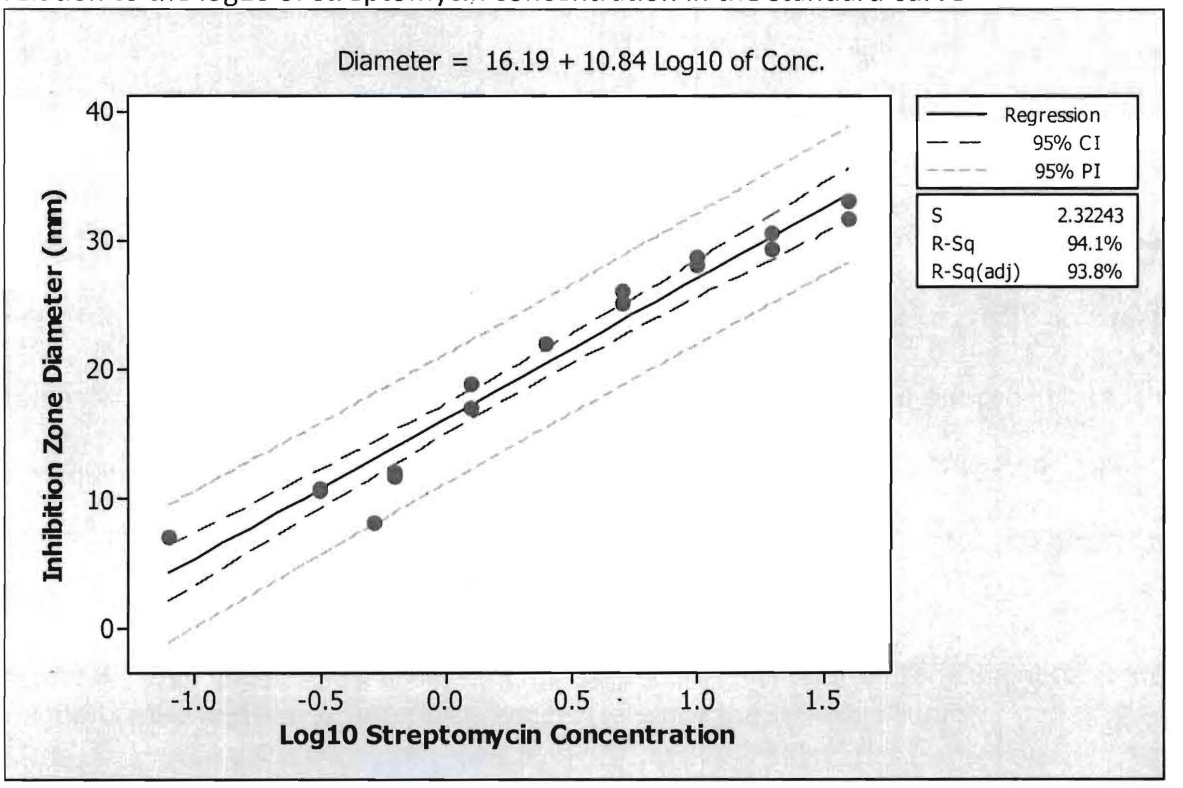


*Each of the treatments included *S. griseus* plus the challenge microbe, except for the negative control which contained only *S. griseus*

The same methods were conducted on the data collected regarding streptomycin production by *S. griseus* and its co-cultures. The inhibition zone diameters themselves were smaller than those observed with cycloheximide – even though streptomycin is the antibiotic *S. griseus* is best known for.

The data from the standard curve for streptomycin was, again, used to conduct a linear regression analysis. This data was also used to construct a best fit like to create an equation that was used to determine the actual streptomycin concentrations produced in by each treatment based on the diameter of their inhibition zone (Figure 6).

Figure 6. Fitted line plot from linear regression analysis of inhibition zone diameter (mm) in relation to the log10 of streptomycin concentration in the standard curve



Once the equation shown in Figure 6 was used to determine the streptomycin concentrations of all the treatment groups, the tests for normal distribution and equal variance were conducted on the antibiotic concentration data. Figures 7 and 8 show that the data

passed the normal distribution requirement and Figure 9 shows the data for streptomycin displays equal variance, so the One-Way ANOVA was conducted.

Figure 7. Probability plots of streptomycin production by *S. griseus*, *S. griseus* + *StreptoBlue2,* and *S. griseus* + *Strepto8*

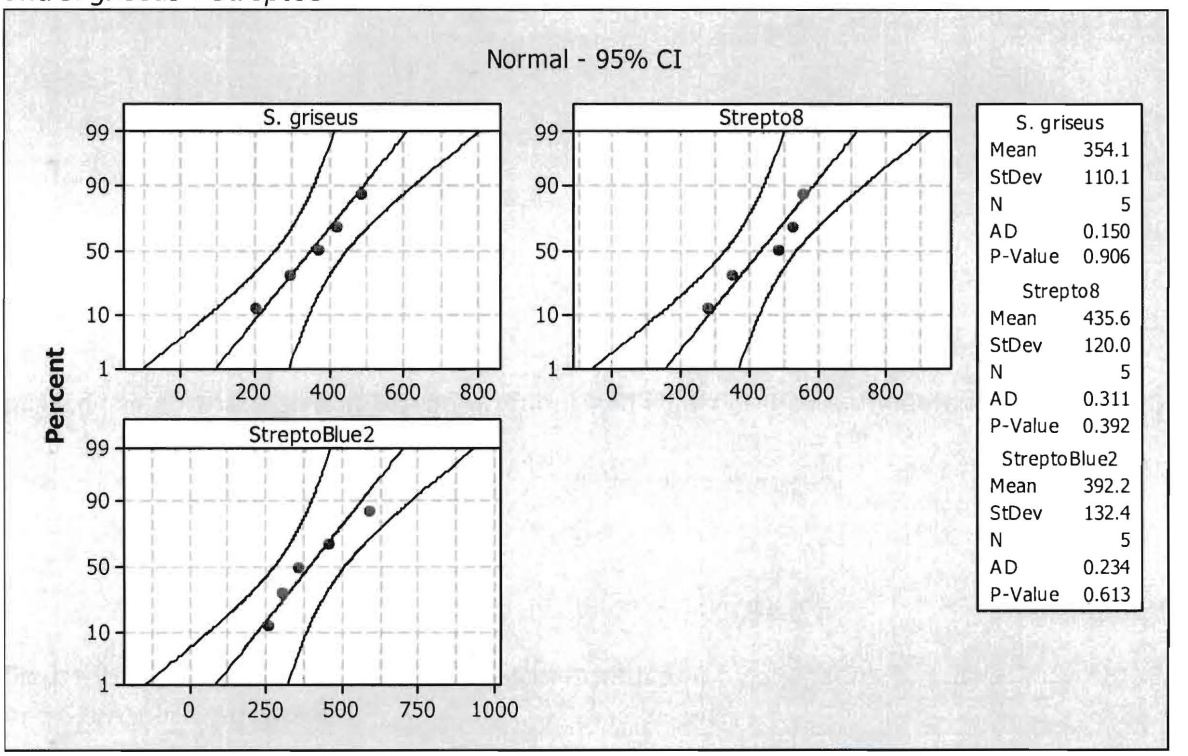
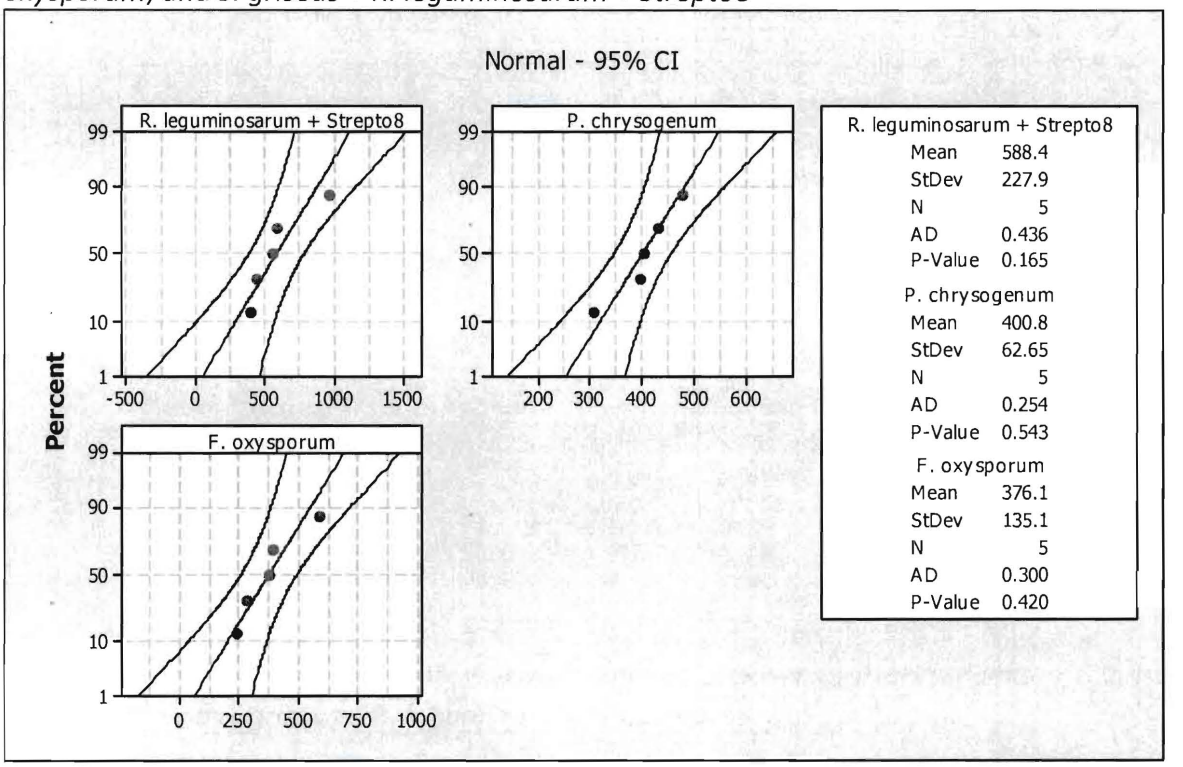
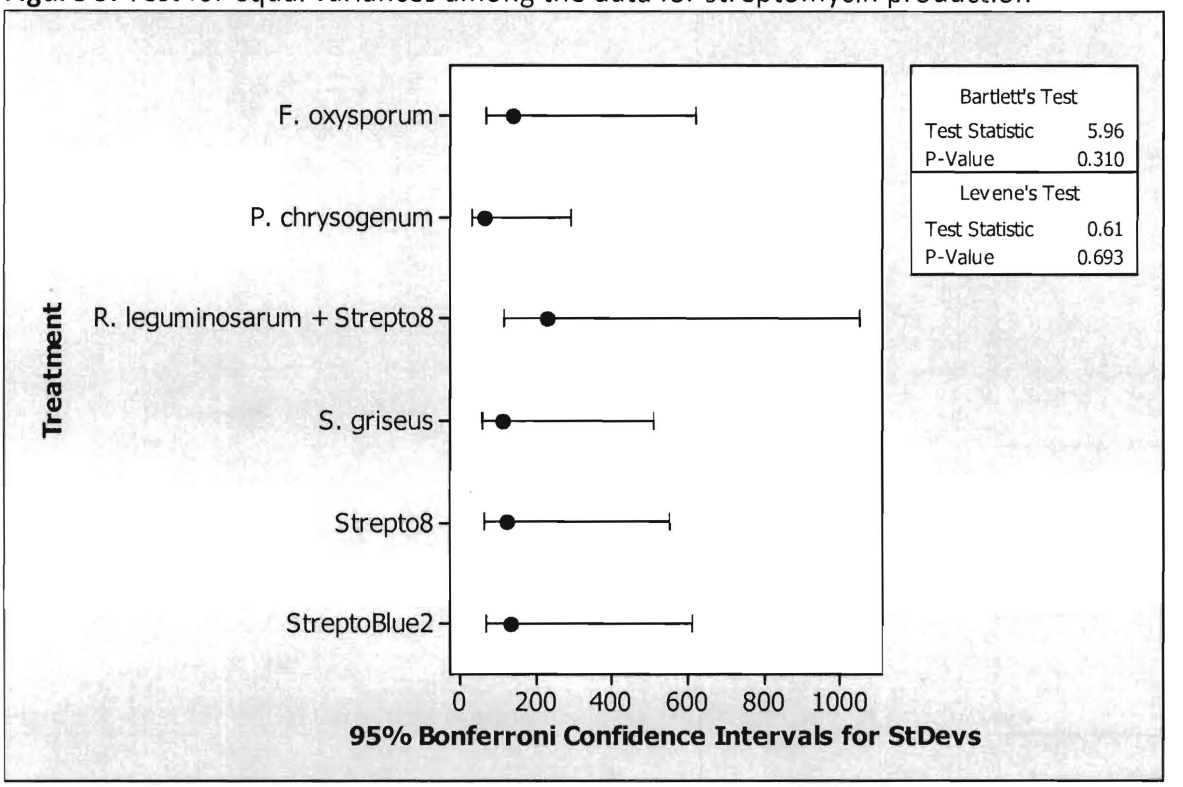


Figure 8. Probability plot of streptomycin production *S. griseus* + *P. chrysogenum*, *S. griseus* + *F. oxysporum*, and *S. griseus* + *R. leguminosarum* + *Strepto8*



*For P-values that are above 0.10 the data is considered to be normally distributed

Figure 9. Test for equal variances among the data for streptomycin production



*The Bartlett's and Levene's tests both test for the Null hypothesis that all group variances are equal by providing a P-value that reflects the Null hypothesis.

According to Figure 9, the P-values for both the Bartlett's Test and Levene's Test were greater than 0.10, so there was no evidence to disprove the Null hypothesis and it was safely assumed there were equal variances among the data for streptomycin. The One-Way ANOVA test was conducted using the data collected for streptomycin production.

Table 2. Results of streptomycin production by *S. griseus* + challenge microorganisms from One-Way ANOVA.

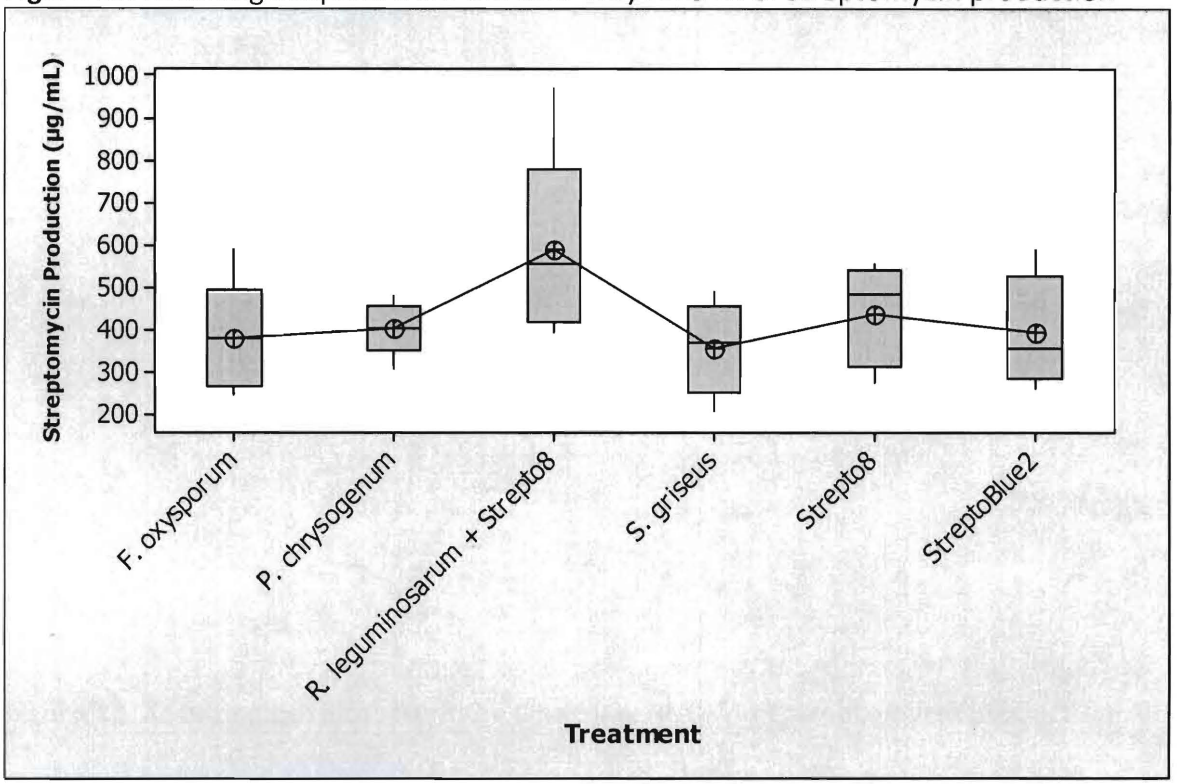
Treatment	N	Mean (µg/mL)	Tukey	Fisher/LSD
S. griseus + R. leguminosarum + Strepto8	5	588.4	A	A
S. griseus + Strepto8	5	435.6	A	A B
S. griseus + P. chrysogenum	5	400.8	A	B
S. griseus + StreptoBlue2	5	392.2	A	B
S. griseus + F. oxysporum	5	376.1	A	B
S. griseus	5	354.1	A	B

*For Tukey and Fisher/LSD tests, each group with a different letter is significantly different ($P \leq 0.05$) from another

The One-Way ANOVA test results are shown in Figure 1. According to the Fisher/LSD portion of the ANOVA, there was only one treatment that was able to significantly increase the production of streptomycin as a co-culutre - *S. griseus* + *R. leguminosarum* + *Strepto8*. The Tukey portion of the ANOVA found that none of the treatments led to a significant increase in streptomycin production. In fact, The Tukey's test did not mark any improvement of streptomycin production, whereas the Fisher/LSD portion showed the *S. griseus + Strepto8* co-culture as having some effect on antibiotic production (Table 2).

Figure 10 shows the boxplots from the ANOVA test that display a better representation of the lack of significant increase in streptomycin with the use of these co-cultures.

Figure 10. Resulting boxplots from the One-Way ANOVA of streptomycin production



*Each of the treatments included *S. griseus* plus the challenge microbe, except for the negative control which contained only *S. griseus*

Discussion

This particular experiment was conducted to determine whether co-cultures had an effect on the antibiotic production by *S. griseus*, and if they did, which microorganisms stimulated the best production of the antibiotics streptomycin and cycloheximide. When looking at the results of the One-Way ANOVA, it was clear that the co-cultures used in this experiment did have an effect on the production of antibiotics. The different challenge microbes used here seemed to have a larger effect on the production of cycloheximide rather than streptomycin because there were three co-cultures that were able to produce a significant increase in antibiotic production over S. griseus alone (according to the Fisher/LSD portion of

the One-Way ANOVA): *S. griseus* + *P. chrysogenum*, *S. griseus* + *StreptoBlue2*, and *S. griseus* + *Strepto8* (Table 1).

The same challenge microbes and co-cultures were used to analyze the possible enhancement of streptomycin production by *S. griseus*, but there were slightly different results. It seemed that streptomycin production overall lacked in comparison to cycloheximide synthesis, and it definitely lacked in its ability to have significant increase in antibiotic production over *S. griseus* alone. The Fisher/LSD portion of the ANOVA only showed one co-culture that had a significant increase over *S. griseus*: *S. griseus* + *R. leguminosarum* + *Strepto8* (Table 2). The Tukey portion of the ANOVA test not only determined that there were no treatment groups that had a significant increase, but the results show there was not enough of an increase in general to separate any of the groups partially from *S. griseus*.

Considering the co-culture that produced the least significant increase in cycloheximide production produced the only significant increase in streptomycin production, it is evident that there are different mechanisms taking place in the action of enhancing or even promoting the pathways that lead to the synthesis of these two antibiotics. Investigation into the further understanding of these mechanisms as well as the by-products that these challenge microbes produce will enable a better understanding of the best pairing between antibiotic producing microbe and their challenge microbe.

Future Research

The results from the experimental trials co-culturing *S. griseus* with *Strepto8* reveal the challenge microbe's ability to successfully promote not just one but two antibiotics as secondary metabolites of *S. griseus*. Further research may include investigation into the location and even the specific molecule that is produced by *Strepto8* that acts in inducing the two antibiotic biosynthesis pathways. In order to narrow down possible locations and identities of the inducing molecule of *Strepto8*, a variety of treatments that extends the protocol for previous experiments as well as treatments that test alternative methods (Figure 11).

One of the first questions is whether the molecule is extracellular or intracellular. The traditional protocol requires the challenge microbe must be blended before addition to the *S. griseus* flasks, which means that both intracellular and extracellular molecules will mix. The first step would be to add an additional treatment that is unblended before its addition – if the molecule is intracellular, it will not be able to exert its effects on *S. griseus* in the intact, unblended treatment.

From this point, similar treatments may be applied to both blended and unblended cultures to provide a more in-depth analysis as well as a certain level of replication and control. In order to address whether the stimulating component must be alive to exert its effects on *S. griseus*, portions of both blended and unblended samples should be placed in hot water bath for a sufficient amount of time that leads to death. Further determination regarding the molecule's presence either in the extracellular or intracellular environment may be conducted via a series of centrifugation steps. The supernatant from the first centrifugation of

both blended and unblended cultures can be filtered and used as treatments to determine if this specific molecule is originally present, or excreted into, the extracellular environment (seen with unblended culture) or whether the molecule is initially intracellular and released upon blending. The pellet from this first centrifugation is resuspended and two more centrifugations separated by resuspensions in physiological saline follow. Repeatedly centrifuging the blended and unblended samples serves to wash the material large enough to form a pellet when centrifuged. Using the pellet material that is washed and isolated from the unblended culture as a treatment will help to determine whether the stimulatory molecule is either the intact cells themselves or some mechanism solely involving those intact cells. The pellet material from the blended culture helps to determine if the inducing of antibiotic production is directly a result of some intracellular component or organelle of the cell rather than some small molecule produced within the cell. For any clarification, Figure 11 diagrams the various proposed treatments for the determination of the molecule or component produced by *Strepto8*. All of these treatments serve to narrow the possibilities of the type and location of the specific molecule produced by *Strepto8* that has the ability to induce the simultaneous production of streptomycin and cycloheximide by *Streptomyces griseus*.

The identification and isolation of the specific molecule or component that directs *S. griseus* biosynthetic pathways to produce streptomycin and cycloheximide would unlock the ability to more directly and efficiently promote the production of those antibiotics. This could lead to the direct and calculated use of that particular molecule from *Strepto8* to effectively maximize the production of not one but two antibiotics possibly in a shorter time frame than the current method.

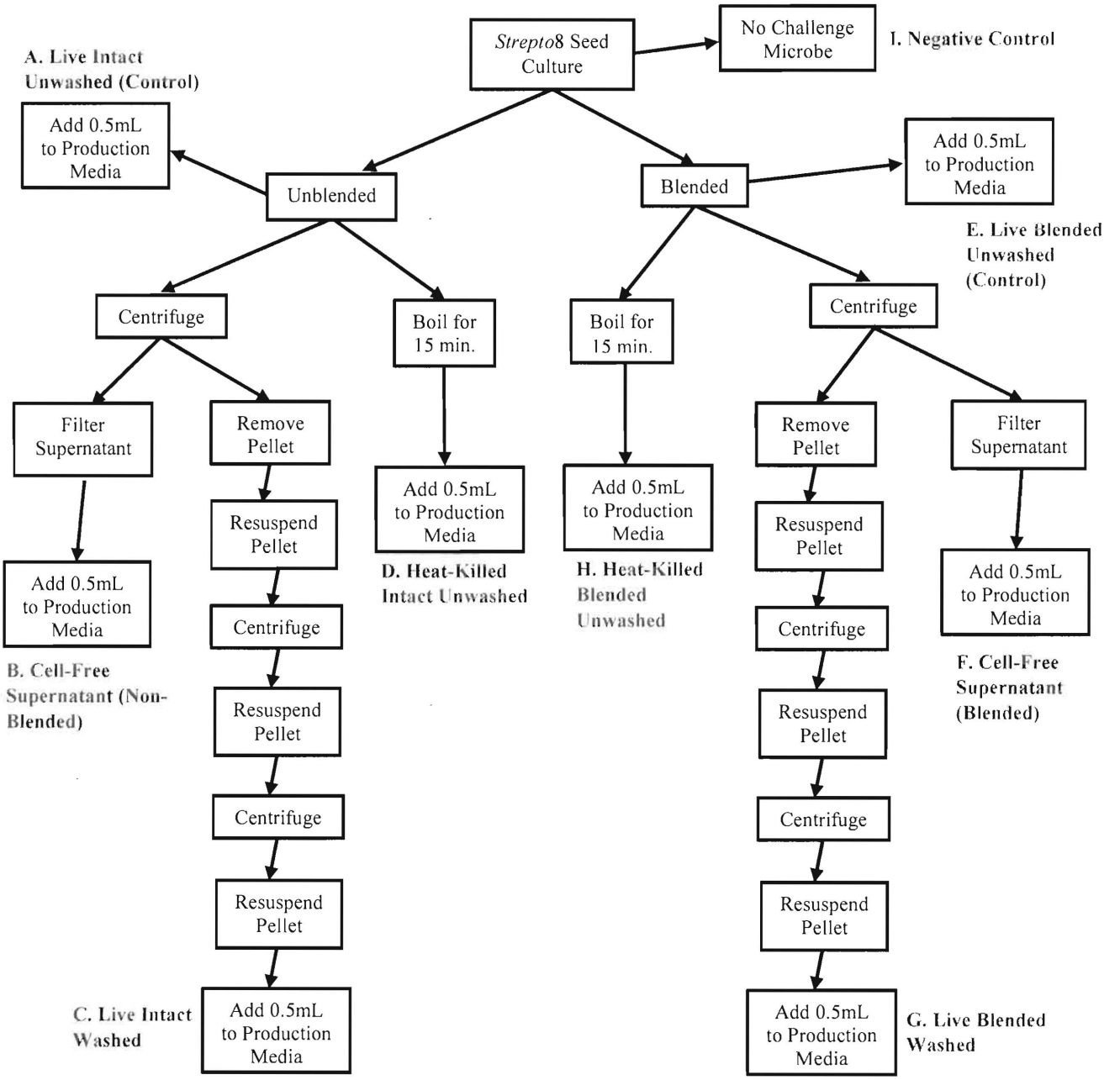


Figure 11. Diagram of proposed treatments for the determination of the location and type of molecule produced by *Strepto*8 that induces the biosynthesis pathways in *Streptomyces griseus* that lead to secondary metabolites streptomycin and cycloheximide

Conclusion

The use of bacterial secondary metabolites in the modern world is vast, but the specific use of antibiotics from secondary metabolism holds great significance in medicine's battle against infectious diseases. The isolation of all antibiotics originates from research into the secondary metabolism process of microorganisms. Considering many of the pathogens encountered today are developing resistances to current antibiotics, understanding the mechanisms behind the induction of secondary metabolism, the conditions required to promote varying secondary metabolites, and any processes that lead to the optimization of production and isolation of these products is more important than ever. Streptomyces griseus is a particular bacterium of interest in this research due to its previously discovered ability to not only produce a number of different antibiotics, but also its ability to produce antibiotics with antibiotic and antifungal actions. Further research should be conducted in the understanding of its already known antibiotic products and the mechanisms of their biosynthetic pathways in order to greatly optimize antibiotic production as well as investigation into other conditions and challenge microbes that may hold the potential to promote the production of novel antibiotics.

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